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**Mycobacteriosis in wild boars: study for the use of
liquid media and antibiotic resistance**

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ABSTRACT

A retrospective study (2004-2010) on 24 *Mycobacterium bovis* strains isolated from wild boars (*Sus scrofa*) (n=18) and cattle (n=6) in Imperia province (Savona province has been included in the study just in 2006) was performed to detect the prevalence and distribution of antibiotic-resistant strains. Actually, in this area cases of *M. bovis* infections in wild boar were related to cases in cattle herds due to the overlapping of their respective habitat and pasture areas, suggesting the role of these animals as bio-indicator of the presence of *M. bovis* in the environment and, in consequence, in farm animals. Susceptibility of all *M. bovis* strains to antituberculous drugs (isoniazid-INH, rifampicin-RIF, ethambutol-EMB and streptomycin-STR) was detected by proportion method on Lowenstein-Jensen (LJ) medium using recommended critical concentration, while the 6 bovine *M. bovis* strains were tested by Versatrek method on liquid medium, too. The proportion method to assess the *M. bovis* susceptibility to INH, RIF, EMB and STR is the gold standard, but the use of ancillary liquid culture systems (Versatrek method) should be performed for the rapid and timely detection of drug-resistance in tuberculosis strains.

All the western Ligurian isolates in this study were sensitive to INH, RIF, EMB and STR by the proportion method; however, light bacterial growth was pointed out in INH medium (2 samples) and in RIF-medium (1 sample). The GenoType® *Mycobacterium tuberculosis*- multidrug resistantplus (MTBDRplus) VER 2.0 assay was used to detect mutations in *rpoB*, *katG*, and *inhA* genes associated with resistance to RIF and INH on the 3 cases of light bacterial growth. No mutation in *rpoB*, *katG*, and *inhA* genes was detected.

The six *M. bovis* isolates from cattle also tested by Versatrek myco susceptibility kit, Versatrek myco streptomycin kit and Versatrek myco PZA kit were fully INH, RIF and EMB sensitive, except for natural pyrazinamide resistance. A single strain of bovine origin showed resistance to STR at lower concentration (2 µg/ml), but this result was not confirmed by the proportion method.

The absence of any drug resistance in cattle could be likely explained with the compulsory eradication program of bovine tuberculosis by elimination of infected animals and the ban on antituberculous drug treatments in animals. However, there is the very real opportunity for other animals in the same herd -which may be harboring tuberculosis- to receive limited antibacterial therapy for other conditions, such as respiratory disease, mastitis, or other localized infections. This may lead to the insurgence of drug resistance in *M. bovis* strains despite all measures adopted for their control. On the other side, considering the feeding habits of wild boars, i.e. rooting, the fact that no drug resistance was found in *M. bovis* strains isolated from wild boars suggests the absence of drug contamination in the environment and confirms their role of bioindicator.

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LIST OF ABBREVIATIONS

ARO	antibiotic resistant organism
ATB	antibiogram
BCG	Bacillus Calmette-Guerin
CFU	colony forming units
DRs	direct repeats sequences
EMB	ethambutol
ESP	ESP® Culture System II
HGT	horizontal gene transfer
INH	isoniazid
LJ	Lowenstein Jensen
MAP	Agar proportion method
MDR	multidrug resistant
MTBC	<i>Mycobacterium tuberculosis</i> complex
OFX	ofloxacin
PCR	polymerase chain reaction
RIF	Rifampin/rifampicin
STR	streptomycin
TB	tuberculosis
VNTR	variable number of tandem repeats
VTI	VersaTREK Automated Microbial detection System
XDR	extensively drug resistant

I. INTRODUCTION

Mycobacterium (M.) bovis is the causative agent of bovine tuberculosis (TB). Therefore, it has received special consideration in livestock owing to the economic impact of infections in this context. Moreover, *M. bovis* can infect a wide variety of hosts, including wild animals, captive species, primates. Such disease is an important zoonosis which poses a significant threat to humans as it can be transmitted through consumption of contaminated milk and close contact with infected domestic or wild animals. The presence of the bovine tuberculosis in wildlife is generally not easily controllable and it raises problems of public health, wildlife management and may interfere with the eradication programmes. The presence of sympatric infected wild species in areas with TB-infected cattle herds (Delahay *et al.*, 2002; Witmer *et al.*, 2010), in fact, is thought to be one reason for the failure of several European countries (Ireland, Italy, Portugal, Spain and the UK) to obtain an official tuberculosis free (OTF) *status* set out by the World Organization for Animal Health (OIE) (www.oie.int/fileadmin/Home/eng/Health_standards/tahc/2010/en_chapitre_1.11.6, 03/02/2012).

In humane medicine TB has remained a significant cause of morbidity and mortality in resources-poor nations (Corbett *et al.*, 2003), but currently threatens to re-emerge in developed nations as well due to its synergy with HIV/AIDS, demographic changes and subsequent immigrations (Davies 2003). Moreover, an emerging trend in the incidence and resistance of *M. bovis* in humans, which has important consequences for its management, was reported in Ireland since 2008; 28.5% were resistant to both pyrazinamide and isoniazid (McLaughlin *et al.*, 2012). In a report from San Diego in 2003 and New York in 2005, 7 and 9% of *M. bovis* isolates were noted to be resistant to pyrazinamide and isoniazid, respectively (Lobue *et al.* 2003; Centers for Disease Control and Prevention, 2005). Two cases of *M. bovis* resistant to isoniazid, rifampicin, and pyrazinamide were reported in Scotland in 1998 (Armstrong *et al.*, 1998). Higher resistance rates have been noted in cattle, too (Sechi *et al.*, 2001; Cavirani *et al.*, 2003).

The first cases of tuberculosis in wild boars in Imperia province have been reported in 1989 (Mignone *et al.*, 1991). The diagnosis was based on anatomopathological examination of hunted wild boars and confirmed by microbiological methods, biological assay and histopathological examination. Since 1995 the submandibular and retropharyngeal lymph nodes of hunted wild boars in Imperia province were examined for tubercular lesions and a large amount of data was collected.

A total of 2,826 lymph nodes, harbouring lesions suggestive of tuberculosis, were collected from 2000 to 2011. Fifty-eight out of 2,826 were positive for *M. bovis* (2%), 179 for *M. microti* (6%). Wild boars, in fact, may be infected by other Mycobacteria, e.g *M. microti*, probably by rooting and eating infected dead small rodents.

M. bovis spoligotypes isolates observed from the wild boars hunted between 2000 and 2005 in Imperia province had the same pattern as some bovine isolates, confirming the hypothesis that wild boar represents a sensitive indicator of environmental contamination by *M. bovis* (Dondo *et al.*, 2006). During 2006 hunting season in Savona province, 13 out of 75 wild boars observed showed tuberculous lesions; of these, 4 belonged to spoligotype ETR A-E 54433, the same isolated from cattle commingling in the same area (unpublished data). The aggregation of wild boars at a single available watering site, as well as supplemental feeding of animals like in this case, could be associated with an increasing risk of detecting TB lesions in this species in that area. This is probably related to the enhanced opportunity for transmission from infected cattle herds, as reported by Miller and Sweeney (2013) in North American wildlife.

Except for this wildlife monitoring activity limited to the single province, until 2012 Liguria region hasn't performed a regional wildlife monitoring program. Only in 2013 a 2-years wildlife monitoring project extended to the all regional territory was funded.

Wild boar represents a sensitive indicator of environmental contamination by *M. bovis* in Imperia province, and monitoring antimicrobial resistance in these

animals in a long term period is very useful to detect any emerging antibiotic resistant pattern. However, we should remember that the reason why drug-resistant strains of *M. bovis* are not emerging in animals nearly as rapidly as in human populations is because of the lack of treatment-related selection pressures. No one is suggesting that individual cattle or wild boars which are identified as tuberculosis suspects should start to receive antimicrobial therapy as we do with infected people. When whole-herd test and individual animal culling practices are employed, there is the very real opportunity for other animals in the same herd which may be harboring tuberculosis, to receive limited antibacterial therapy for other conditions, such as respiratory disease, mastitis, or other localized infections.

In this type of environment, asymptomatic and undetected tuberculosis carriers will be subjected to similar selection pressures that have produced the current worldwide emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) tuberculosis strains in humans. This is not meant to find fault with newer management procedures for bovine tuberculosis but only to point out that selection pressures which promote antimicrobial resistance development may well be increasing, at least in our domestic and captive animal species. Therefore, continued surveillance for drug-resistant strains of *M. bovis* will be even more important as we move forward to ensure the safety of our milk, beef and wild boar meat sources for human consumption.

For all these reasons, the current study has characterized the drug resistance pattern of *M. bovis* strains isolated from cattle and wild boars living in the same area (Imperia province, Liguria region, Italy) with a view to evaluate the use of different antibiotic susceptibility techniques (microbiological and molecular) with both liquid and solid media.

1.1 Mycobacterium bovis and the Mycobacterium tuberculosis complex

M. bovis, the causative agent of bovine tuberculosis, can also cause disease in a variety of domestic and wild animals. *M. bovis* is a member of the *M. tuberculosis* complex (MTBC).

MTBC includes the typical human-associated pathogens *M. tuberculosis* and *M. africanum* (de Jong *et al.*, 2010), *M. canettii* and the so called “smooth TB bacilli” (Gutierrez *et al.*, 2005), the actual host range of which remains unknown, and several lineages adapted to different mammal species that includes *M. bovis* which is responsible of bovine tuberculosis and includes the vaccine strain *M. bovis* BCG, *M. microti*, *M. caprae*, *M. orygis* and *M. pinnipedii* (Brosch R. *et al*, 2002; Huard *et al.*, 2006; van Ingen J *et al.*, 2012). Of note, novel members of MTBC affecting wild mammals in Africa have recently been discovered (Cousins *et al.*, 1994; Alexander *et al.*, 2010).

1.2 The role of Wildlife for Bovine Tuberculosis

Worldwide, wildlife plays an important role in TB epidemiology (Zumarraga *et al.*, 1999; Artois *et al.*, 2001; Schmitt *et al.*, 2002; Fitzgerald and Kaneene, 2013). Domestic and non-domestic animals may be considered either as maintenance (or reservoir) hosts or non-maintenance (or spill-over) host for bovine tuberculosis. In reservoir host species, infection can persist through horizontal transfer in the absence of any other source of *M. bovis* and may as well be transmitted to other susceptible hosts. In contrast, spillover hosts become infected with *M. bovis* but the infection only occurs sporadically or persist within these population if a true maintenance host is present in the ecosystem. If the source of infection is removed, the prevalence for this disease is reduced and it can only be maintained in the long term from another source (Haydon *et al.*, 2002). Extensive investigations of sporadic *M. bovis* reoccurrence have shown that wildlife *reservoirs* exist in some countries and can act as a source of infection for cattle, deer and other livestock. The role of different potential host species may vary geographically with habitat, ecology,

land use and consequently behaviour (Gortàzar *et al.*, 2011). The potential for transmission to the cattle population was evaluated by Hardstaff *et al.*, 2014 across Europe by determining the level of TB hazard for which a given wildlife species is responsible. Wild boars posed the greatest hazard of all the wildlife species in Europe, indicating that these animals have the greatest ability to transmit the disease to cattle.

In Europe, the most well known case of maintenance host is that of the badger (*Meles meles*) in the United Kingdom and the Republic of Ireland (Willesmith, 1991; O'Reilly & Daborn, 1995; Corner, 2011), but TB cases have repeatedly been reported in other mammals, including wild and semi domestic (i.e. fenced-in) cervids (Aranaz *et al.*, 1996; Prodingler *et al.*, 2002), and even the wild boar (Serraino *et al.*, 1999; Dondo *et al.*, 2007; Naranjo *et al.*, 2008) and the brush-tail possum (*Trichosurus vulpecula*) in New Zealand (Animal health division-New Zealand-1986). Known wildlife *reservoirs* of *M. bovis* in North America include white-tailed deer (*Odocoileus virginianus*) in both Michigan and Minnesota, United States, and both elk (*Cervus canadensis*) and white-tailed deer in Riding National Park, Manitoba, Canada (table 1).

Nevertheless, the role of European wild ungulates in the epidemiology of TB is still under discussion. Wild boars (*Sus scrofa*) are considered to be reservoir hosts in Spain (Naranjo *et al.*, 2008), but spill-over hosts in Norwest-Italy (Serraino *et al.*, 1999; Dondo *et al.* 2007). The role of wild boar varies depending on the local epidemiological context and wildlife management practices. In fact, the host status of a species with regard to *M. bovis* may differ between regions or may change over time depending on population density or management regimen (Cousins and Florison, 2005). Due to the low frequency of detection of generalized tuberculosis in *M. bovis* infected European wild boars (*Sus scrofa*), and to the fact that infected wild boars have only been detected in areas with infected cattle, Serraino *et al.* (1999) hypothesized that wild boars are the end host for *M. bovis* infection, with little transmission possibilities to livestock and little relevance as a *reservoir*. This hypothesis was confirmed by Dondo *et al* (2007), who pointed out that *M.*

bovis prevalence in wild boar is related to its prevalence in cattle herds due to the overlapping of their respective habitat and pasture areas, suggesting the role of these animals as bio-indicator of the presence of *M. bovis* in the environment and, in consequence, in farm animals. Western Liguria, the geographical area that was the focus of our study, is not officially free of bovine tuberculosis by the comunitary law. In 2010, the Italian Ministry of Health, reported that the overall proportion of farms with positive cattle in Liguria reached 0,2% (Italian Ministry of Health, 2010).

The detection of infection in a wildlife population requires bacteriological investigation or the use of a valid testing method for the species involved together with epidemiological analysis of information.

Table 1. Summary of Key Features of Bovine Tuberculosis in Wildlife Reservoirs (according to Fitzgerald and Kaneene, 2013).

	Reservoir Host					
	White-tailed Deer and Elk	European Badger	Wild Boar	Brush-tailed Possum	African Buffalo	Lechwe
Geographic location	North America	England and Ireland	Spain	New Zealand	National parks in South Africa	Kafue Basin, Zambia
Significance of reservoir	Spillover into domestic cattle	Spillover into domestic cattle	Spillover into domestic cattle, goats, deer, and pigs	Spillover into domestic cattle, deer, and ferrets	Spillover into other wildlife species	Lechwe population decline
Gross lesion distribution	Cranial lymph nodes, lungs	Lungs, lymph nodes, subcutaneous bite wounds	Cranial lymph nodes; common dissemination	Lungs, lymph nodes	Lymph nodes of head and thorax	Lungs and pulmonary lymph nodes
Important disease features		Latency, NVL		Draining fistulous tracts, NVL		Diaphragmatic lung lobes
Contributing factors	Association with cattle; high population density	High population density	Increasing population	Introduction of reservoir to New Zealand	Association with cattle; highly gregarious host	High correlation with liver fluke infestation
References	4, 5, 14, 22, 28, 31, 32, 33, 43, 44, 45	8, 10, 11, 13, 16, 17, 20, 34	1, 18, 19, 23, 27, 30, 38, 41	2, 3, 6, 7, 12, 35, 36, 40	21, 24, 25, 37, 39	15, 26

NVL, no visible lesions are common.

1.3 The antibiotic resistance

In general, antimicrobial resistance is the capacity of a microorganism to resist the growth inhibitory or killing activity of an antimicrobial beyond the normal susceptibility of the specific bacterial species (McDonnell and Russell, 1999; Acar *et al.* 2011; Mathur *et al.* 2005). Resistance to an antibiotic typically develops from its use and is a classic example of Darwin's principle, "survival

of the fittest". The epidemiology of antibiotic resistance is made more complicated by the ability of genes responsible for such resistance to spread between different types of bacteria. Also, antibiotic-resistant bacteria can spread across sectors, settings and geographical borders. This spread can be attributed to people, animals, animal products or environmental contamination. Some bacteria have been resistant since ancient times, and are said to have natural or intrinsic resistance. In other cases, susceptible bacteria have become resistant over the course of the last several decades: acquired resistance. Bacteria are remarkably resilient and adaptable and can change rapidly in response to a change in their environment, such as the presence of an antibiotic. Three types of resistance are described. Microbiological resistance (*in vitro* resistance) means a reduced susceptibility of bacteria to antibiotics above a breakpoint that is defined by the upper limit of normal susceptibility of the concerned species, which is also called epidemiological resistance. The microbiological resistance can often be confirmed genotypically by demonstrating the presence of a certain antimicrobial resistance gene or resistance mechanism via molecular techniques. Secondly, there is the pharmacological resistance. This is based on pharmacokinetic parameters and the normal susceptibility of a bacterial species. If the minimal inhibitory concentration (MIC) of the antibiotic for the bacteria concerned is within the concentration range that can be attained by that antimicrobial, it is susceptible. If the MIC of the antibiotic for the concerned bacteria is higher than the concentration that can be attained at the site of infection, then the bacterium is regarded as resistant. Finally, clinical resistance (*in vivo* resistance) means an infection with the concerned bacterium cannot be treated appropriately anymore and treatment failures are evident (Van Eldere, 2005). Bacteria can be resistant to antibiotics by using several mechanisms: enzymatic degradation of antibiotics, antibiotic target modification, changing the bacterial cell wall permeability and alternative pathways to escape the activity (Verraes *et al.* 2013).

A susceptible bacterium can become resistant through a novel genetic mutation in its DNA (chromosomal resistance) or, more commonly, through the acquisition of mobile genetic elements from another bacterium that is already resistant (horizontal gene transfer) (Fig. 1). There are three main mechanisms of horizontal gene transfer (HGT) between bacteria: conjugation, transformation and transduction. These may occur in the soil, in water, in the digestive system of humans and animals, as well as in food (Verraes *et al.* 2013). HGT of antimicrobial resistance genes, their maintenance in bacterial populations and the creation of multidrug resistance is greatly enhanced by genetic structures such as plasmids, integrons and transposons (Aarestrup, F.M., 2006; Salyers *et al.*, 2007, Bennett *et al.*, 2008; Revilla *et al.*, 2008). These are mobile genetic elements since they represent a pool of mobile DNA. The frequency of HGT largely depends on the properties of the mobile genetic element, the characteristics of the donor and recipient populations, and the environment. Complicating matters is the ability of one resistance gene to often confer resistance to two or more antibiotics that usually belong to the same antibiotic class, so-called *cross-resistance*. Also, different resistant genes that confer resistance to different antibiotic classes are often located together in the DNA of the bacteria and can be transferred simultaneously (referred to as *co-resistance*). Thus, usage of one type of antibiotic can result in resistance not only to this antibiotic but also to others in the same class (*cross-selection*) or in other classes (*co-selection*).

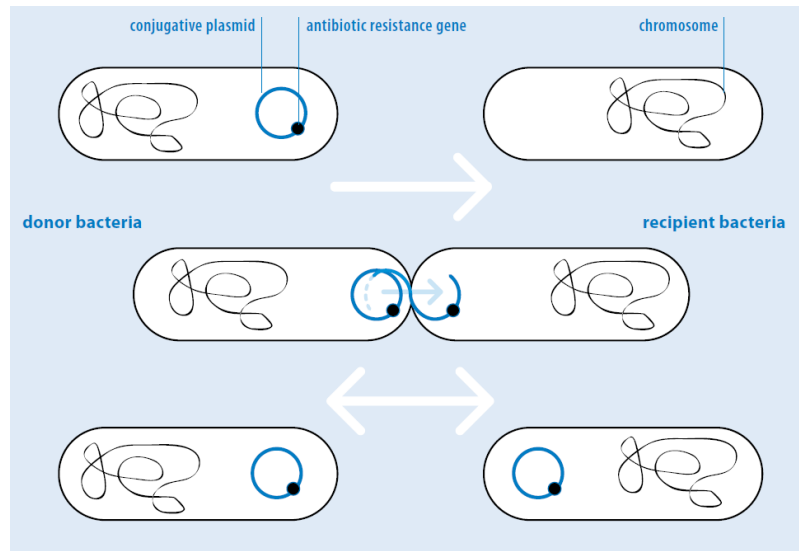


Fig. 1. Horizontal gene transfer: resistance gene being transferred from one bacterium to another (in accordance to World Health Organization regional office for Europe, 2011).

When a bacterium becomes resistant to an antibiotic through a novel mutation in its DNA, the spread of the strain itself is the principal method of spreading the resistance. As bacteria reproduce very rapidly, organisms with this new resistance can rapidly become dominant in a bacterial population within an individual or an animal, particularly if the use of an antibiotic to which the strain is resistant wipes out competing bacteria in its immediate environment.

The subsequent spread of resistant organisms through people or animals, or both, can ensure that the resistant organism becomes widely dispersed.

The consequences of horizontal gene transfer are even more alarming. This mechanism can often promote the simultaneous spread of resistance to several unrelated classes of antibiotics, particularly if the genes for such resistance are co-located on the transmissible genetic element.

Also, there is another important mechanism. When resistance has developed, bacteria may often retain it for long periods in the absence of exposure to antibiotics. This may lead to persistence of resistance to antibiotics that are either rarely or no longer used.

To make matters worse, sometimes genes for resistance and virulence can be transferred together, leading to the emergence of new resistance threats of

greater virulence and pathogenicity than seen in past generations. The mass media often call such pathogens *superbugs*.

The quantitative explosion of resistance from individual cellular events (mutation and/or gene transfer) to global health challenges has relied on two additional aspects:

- (a) antibiotic selection pressure
- (b) demographic and geographic spread.

Since antibiotics kill susceptible bacteria, resistant bacteria have less competition for resources and can flourish, especially when antibiotics are present.

Antibiotic-resistant bacteria can become established and persist, even after antibiotic use is discontinued.

People and animals normally carry vast numbers of diverse bacteria in the gut, on the skin and on other surfaces. Resistant bacteria can be carried by people and animals that are not sick, and transferred between individuals and communities, and around the world from people, animals, food and trade goods that carry them and from waterways (World Health Organization regional office for Europe, 2011).

1.4 M. bovis: a zoonotic and antibiotic resistant pathogen

M. bovis is both the causative agent of TB and a zoonotic pathogen, enclosed in Annex I. A of the Legislative Decree No. 191/2006 implementing Directive 2003/99/EC on the surveillance of zoonoses and zoonotic agents and antibiotic-resistance. The European Union is stepping up monitoring of zoonoses, zoonotic agents and related antimicrobial resistance. It has laid down minimum requirements applicable in the Member States to reinforce their existing monitoring systems, through which they collect, analyze and disseminate data on these phenomena with a view to identifying and characterizing hazards, assessing exposure and defining the associated risks.

Zoonotic diseases are responsible for most (60.3%) emergent diseases of humans. Moreover, the preponderance (71%) of emerging pathogens is of

wildlife origin or has an epidemiologically important wildlife host (Jones *et al.*, 2008). Wild animals are susceptible to infection by many of the same pathogens that afflict domestic animals, and transmission between domestic animals and wildlife can occur in both directions. One such pathogen is *M. bovis*, the causative agent of tuberculosis in cattle and most other mammals, wild and domestic.

The disease is an important zoonosis which poses a significant threat to humans as it can be transmitted through consumption of contaminated milk and close contact with infected domestic or wild animals. The biggest concern for human exposure is to those hunters who field-dress wild boar carcasses and come into contact with tuberculous lesions.

Control of transmission from the wildlife population to farmed species is complex and, to date has relied on the reduction or eradication of the infected wildlife population.

On the other hand the increasing prevalence of antibiotic resistant strains of bacteria in both animals and humans is an emerging public health issue.

Of particular concern is bacterial resistance in wild animal populations due to antibiotics commonly used in human and veterinary medicine, aquaculture and agriculture (Fig. 2).

Mycobacterial species in the *M. tuberculosis* complex undergo low-frequency spontaneous chromosomal mutations which result in genetic resistance to antituberculosis drugs. Spontaneous mutation in *M. tuberculosis* isolates resulting in drug resistance occur at reported frequencies between 1.0×10^{-7} and $2.95 \times 10^{-8.6}$. For example, *M. tuberculosis* undergoes spontaneous mutations resulting in resistance to isoniazid at a frequency of 3.5×10^{-6} and mutations resulting in rifampin resistance at a frequency of 3.1×10^{-8} (Johnson *et al.*, 2006).

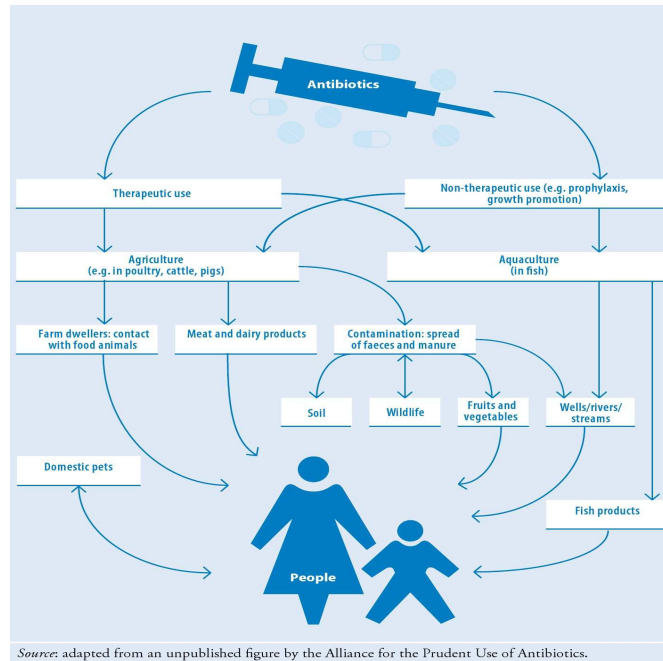


Fig. 2. The flow of antibiotic-resistant bacteria (in accordance with World Health Organization regional office for Europe, 2011).

The proliferation of antibiotic-resistant organisms (ARO) in the environment reflects the extension of resistance beyond the hospital setting and likely indicates multiple sources of exposure and transfer of resistant strains to nontarget population. Genetic studies have demonstrated that some mutations in target genes are associated with resistance to isoniazid (*katG*, *inhA* and the intergenic region *oxyR*–*ahpC*), rifampicin (*rpoB*), streptomycin (STR) (*rpsL*, *rrs*), ethambutol (EMB) (*embB*) and quinolones (ofloxacin, OFX) (*gyrA*, *gyrB*) (Telenti *et al.*, 1993; Honore *et al.*, 1994; Takiff *et al.*, 1994; Telenti *et al.*, 1997; Ramaswamy *et al.*, 2003). Most mutations have been described in *Mycobacterium tuberculosis* strains and limited data are available regarding the genetic assessment of drug-resistant *M. bovis* strains (Blázquez *et al.*, 1997), in particular *M. bovis* strains isolated from animals, and only genes involved in INH and RIF resistance have been further studied (Sechi *et al.*, 2001; Blázquez *et al.*, 1997; Samper *et al.*, 2005). *M. bovis* isolates are naturally resistant to pyrazinamide, a first-line anti-TB drug, due to a single C-G point mutation at nucleotide 169 at the *pncA* gene (Scorpio *et al.*, 1996). A

serious concern is the development, in human medicine, of MDR strains of mycobacteria, resistant at least rifampicin and isoniazid (first-line antituberculosis drugs) and XDR strains of mycobacteria, resistant to rifampicin and isoniazid and second-line antituberculosis drugs (the fluoroquinolones and either amikacin, kanamycin or capreomycin) (Jain and Dixit, 2008).

In humans, considerably fewer cases of TB are caused by *M. bovis* than *M. tuberculosis* (Pérez-Lago *et al.*, 2013); *M. bovis* accounts for approximately 0,5-1,5% of the entire human TB cases in some developed countries (Chen *et al.*, 2009); nevertheless, diagnostic limitations mean that currently available data on prevalence grossly underestimate the true dimension of the problem. There are many reports of MDR strains of *M. bovis* emerging in humans in Europe and throughout other less developed countries throughout the world (Cobo *et al.*, 2001; Gomez-Lus *et al.*, 2000). Although development of MDR strains of *M. bovis* is less frequently reported than MDR strains of *M. tuberculosis*, it is still a problem, especially in countries that continue to have high rates of *M. bovis* infection in their milking goats and cattle (Mukherjee *et al.*, 2004) and the outbreaks caused by these strains have had a dramatic impact (Dankner *et al.*, 1993; Guerrero *et al.*, 1997; Nitta *et al.*, 2002; Hughes *et al.*, 2003). These infections are especially problematic in immunosuppressed people as well as in hospital settings serving as nosocomial infections (Cobo *et al.*, 2001; Mukherjee *et al.*, 2004). Despite this fact, few studies have been developed to test susceptibility of *M. bovis* from animal origin (Sechi *et al.*, 2001; Cavirani *et al.*, 2003; Hughes *et al.*, 2003; Parreiras *et al.*, 2004; Daly *et al.*, 2006; Romero *et al.*, 2007; Fitzgerald *et al.*, 2011; Silaigwana *et al.*, 2012).

Multidrug resistant strains of *M. bovis* have been identified worldwide, and, the ease and the frequency of human foreign travel, international spread of these strains is of concern.

Fortunately these forms of antituberculosis drug resistance are not as prevalent in veterinary medicine as they are in human medicine given the compulsory

eradication program of bovine tuberculosis by elimination of infected animals and the ban on antituberculous drug treatments in animals. In fact, positive domestic animals are slaughtered, not treated and released, and wildlife positives are always detected following harvest of the affected animal.

Therefore fraudulent actions are reported, and common drugs, like streptomycin, are effective against tuberculosis too. For this reason the possible detection of resistant *M. bovis* strains appears of great interest.

II. THE PRESENT INVESTIGATION

2.1 PURPOSE OF THE STUDY

The aim of this study consists in a retrospective investigation (2004-2010) to evaluate the prevalence and distribution of antibiotic-resistant *M. bovis* strains (isoniazid, ethambutol, streptomycin and rifampin) cultered from cattle and wild boars (*Sus scrofa*) living in the same area (Imperia province), and improve the use of most rapid and sensitive diagnostic tests that provide reliable results.

Rapid diagnosis of tuberculosis is important in human medicine for initiation of appropriate drug therapy and institution of measures to prevent the spread of this highly contagious disease. Delayed treatment, particularly of multiple-drug resistant *M. tuberculosis* (MDR-TB) strains, can result in treatment failure and patient mortality (Stratton, 1992). On the other hand, in veterinary medicine, the detection of antibiotic resistant *M. bovis* strains allows to investigate the likely role of animals as source of drug-resistant human cases. Regarding mycobacterial testing, experts at the Centers for Disease Control (CDC) have made several recommendations, including the use of both liquid and solid medium for mycobacterial culture (Tenover *et al.*, 1993).

2.1.1 Specific objectives

- i. To detect any evidence of new antimicrobial resistant *M. bovis* strains to isoniazid, ethambutol, rifampin and treptomycin.
- ii. To use the gold standard proportion method to detect susceptibility of *M. bovis* strains to isoniazid, rifampin, ethambutol and streptomycin.
- iii. To perform tests of antibiotic resistance to specific drug concentration by liquid culture system (VersaTREK Myco susceptibility kit, Streptomycin and PZA tests).
- iv. To use molecular techniques for fast detection of mutations in *rpoB*, *katG*, and *inhA* genes associated with resistance to RIF and INH.

- v. To evaluate the role of wild boar as indicator of drug contamination in the environment.

2.2 MATERIALS AND METHODS

The summary of the general methods is reported below:

2.2.1 Study area and samples

2.2.1.1 Imperia province

The study area was limited to Imperia province (Savona province has been included in the study just in 2006) (fig 3). This probably represents a limitation, but we are planning to extend the survey also to *M.bovis* strains isolated in the entire Liguria region, Piedmont and Valle d'Aosta region.

The hunting area is managed according to the division of the territory into Ambito territoriale di Caccia unico (A.T.C. Unico di Imperia) and Comprensorio Alpino di Caccia. The total surface of Imperia A.T.C. is of 75,171.61 ha, the Comprensorio alpino has a total extension of 33,470.30 ha.

During the year 2011 the number of cattle reported in national data base (<http://statistiche.izs.it>) in Imperia province was 2,727, while the number of wild boars (estimated data-Amministrazione provinciale di Imperia) was 4,500.



Fig. 3 Study area: Imperia and Savona provinces

2.2.1.2 Period of study

The investigation includes a seven-years study (from hunting season 2004 to hunting season 2010) to detect any emerging drug resistance pattern.

2.2.1.3 Samples collection

The samples originated from skin test–positive cattle identified within the national or regional eradication programs, from abattoir surveillance, and from postmortem inspections of wild boars were collected.

*Wild boars (*Sus scrofa*)*

Surveillance of wild boars in Imperia province is based primarily on postmortem sampling (submandibular and retropharyngeal lymph nodes, diaphragm and blood) obtained from hunter-harvested animals.

Anatomo-pathological examination of submandibular and retropharyngeal lymph nodes was performed. Data regarding hunted wild boars were reported by the hunters on a specific form as follows: date, hunting place, sex, estimated age. Granulomatous lesions referred to tuberculosis (Fig. 4) were collected and submitted to culture for the isolation of *Mycobacterium* spp.

Cattle

Lymph nodes and/or other organs showing tuberculous lesions from skin test–

positive cattle slaughtered within the national eradication programs or from abattoir surveillance were also collected.



Fig. 4. Wild boar submandibular lymph node with lesions attributable to Tuberculosis

Commingling of cattle and wildlife is common in this area where domestic and wild ruminants share pasture resources during spring and summer.

2.2.1.4 Selection of strains

This study was performed on a panel of 24 western ligurian *M. bovis* strains:

- 18 isolated from wild boars (*Sus scrofa*) slaughtered during the hunting seasons 2004-2010 (Fig. 5; tab. 2).
- 6 isolated from skin test-positive cattle (fig. 5; tab. 3) within the national eradication programs or from abattoir surveillance and belonging to 2 tuberculosis outbreaks occurring in Imperia province in 2005-2006.

Sex, estimated age and weight of wild boars is shown in table 2, while table 3 shows the characteristic of cows when known.

Tab. 2. Sex, estimated age and weight of wild boars positive for *M. bovis*

N° strain	Sex	Age (years)	Weight (kg)
1	M	1	30
2	F	1-2	20
3	F	1-2	25
4	F	1	30
5	M	2	80
6	M	2	80
7	M	1-2	80
8	M	2	65
9	M	1	20
10	M	1-2	50
11	nd	nd	nd
12	nd	nd	nd
13	nd	nd	nd
14	nd	nd	nd
15	F	nd	80
16	M	nd	nd
17	M	1-2	nd
18	F	nd	nd

*nd= not detected

Table 3: Year, breed, sex and age (months) of cows that tested positive for *M. bovis*

N° Strain	Year	Breed	Sex	Age (months)
1	2005	nd*	nd	nd
2	2005	nd	nd	nd
3	2005	nd	nd	nd
4	2005	nd	nd	nd
5	2005	nd	nd	nd
6	2006	Piemontese	M	18

*nd= not detected

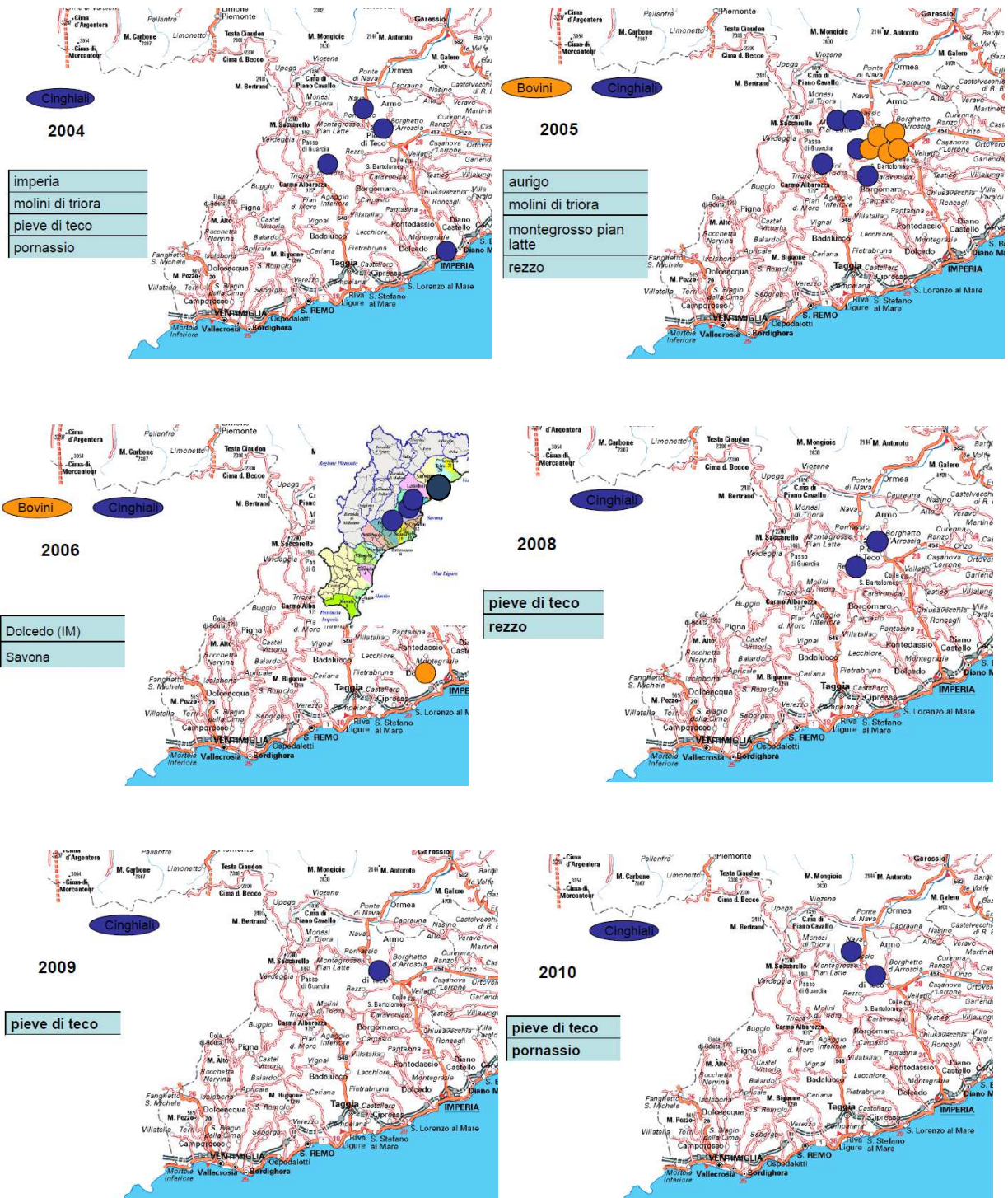


Fig. 5. Number of isolations of *Mycobacterium bovis* in cattle and wild boars (*Sus scrofa*) from 2004 to 2010 in Imperia province (Savona province was included in the study just in 2006).

2.2.2 Samples processing and culture

2.2.2.1 Bacteriological examination for isolation of *Mycobacterium* spp

The flowchart illustrating the diagnostic protocol for the isolation of *Mycobacterium* spp is shown in figure 7.

Connective and fat tissue were firstly removed from lymph nodes samples, then the sample was homogenized and decontaminated in two different ways (Cetylpyridinium chloride 1.5% and Sodium Hydroxide 2%) and both were inoculated onto three different types of solid media: Lowenstein-Jensen, Stonebrink and Lowenstein Jensen w/o glicerol (fig. 6). Solid media were incubated for 10 days to 37°C with 5% CO₂ and then for 80 days to 37°C. Suspected colonies underwent to identification and typing by molecular methods (Dondo *et al.*, 2007).

2.2.2.2 Molecular identification and typing of strains

DNA from isolated strains was extracted by heat shock at 99°C for 20 min in water; identification of isolates was performed by using a multiplex PCR, an in house variation of protocols (data not shown) described by Kulski (Kulski *et al.*, 1995) and by Sinclair (Sinclair *et al.*, 1995).

This Multiplex PCR is based on simultaneous detection of different molecular target: RNAr16S sequence, insertion element IS986 and mpt40 gene. Different *Mycobacteria* species are detected by electrophoretic profile of PCR product. The strains classified as *M. tb* Complex, cluster including other members of *Mycobacteria* (*M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis*, *M. bovis* BCG), were going to be typed using Spoligotyping, usually applied for molecular characterization, but very useful to differentiate *M. bovis*/*M. tuberculosis*. Spoligotyping was performed as described by Kamerbeek (Kamerbeek *et al.*, 1997). This technique is based on *in vitro* amplification of the DNA of the highly polymorphic DR genomic locus present in the *M. tb* Complex chromosome; this locus contains multiple, well conserved 36-bp direct repeats sequences (DRs) interspersed with non repetitive spacer sequences. Strains vary in the number of DRs and in the presence or absence

of these spacer sequences and they could be characterised by specific absence/presence by a PCR based reverse line blot method covalently (Isogen, Netherland).

M. bovis strains isolated were further characterized by VNTR typing (ETR A, B, C, D, E) according to Frothingham (Frothingham *et al.*, 1998).

Unidentified strains by Spoligotyping were processed by PCR-based *M. tb* Complex typing method that makes use of *M. tb* Complex chromosomal region of difference deletion *loci*. According to Huard (Huard *et al.*, 2003) three primer pairs (which amplify within the *loci* Rv1510, Rv3120 and IS1561) were run in separate but simultaneous reactions. The pattern of PCR products from all the reactions allowed immediate identification either as *M. tb* Complex and as *M. microti*.

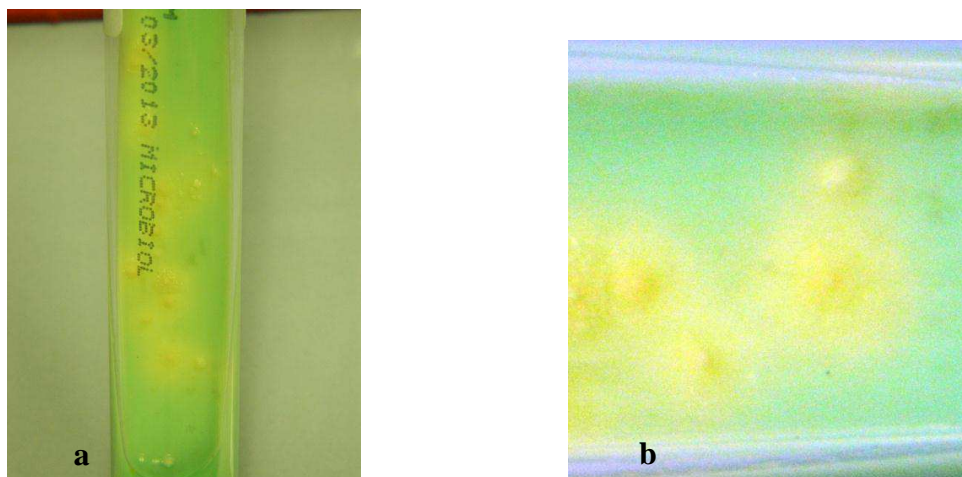


Fig. 6 a. Lowenstein-Jensen medium: primary colony of *M. bovis* **b.** *M. bovis*: higher-magnification image of the colony shown in picture A.

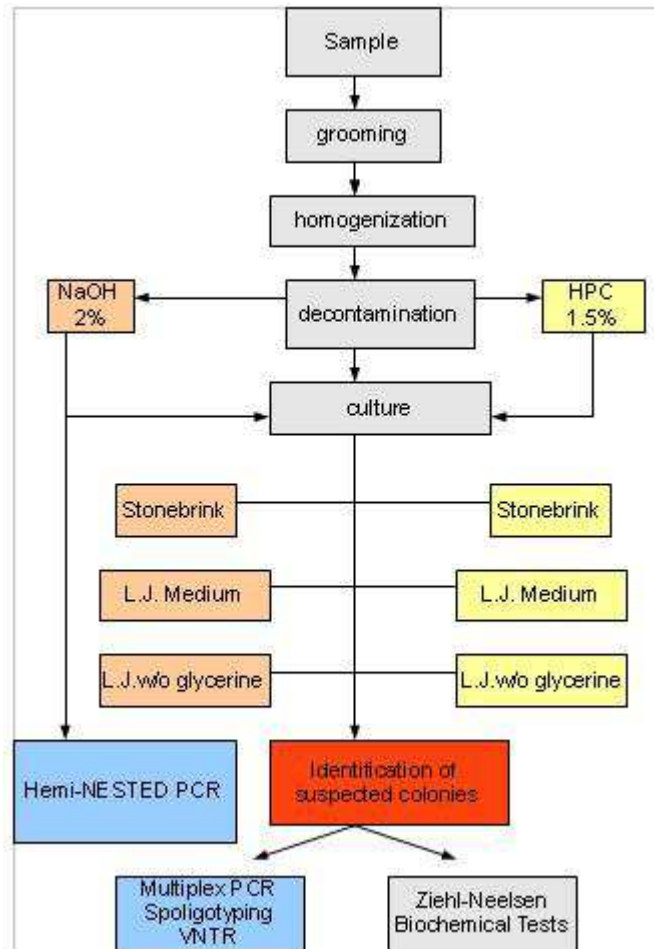


Fig. 7. Flowchart for the isolation of *Mycobacterium* spp.

2.2.3 Drug susceptibility testing

2.2.3.1 Proportion method on LJ medium

The drug susceptibility testing of 24 *M. bovis* isolates was performed following the standard proportion method on Lowenstein-Jensen medium (Fig. 6). Equal amounts of two different dilutions (1:10 and 1:100) of a standardized inoculum (1 McFarland standard=300 x 10⁶ UFC/ml) were inoculated onto agar-based medium with and without the drugs to be tested. Antibiotics were added in different concentrations:

- isoniazid (INH): 0,2 µg/ml
- streptomycin (STR): 2 µg/ml
- rifampicin (RFP): 40 µg/ml
- ethambutol (ETB): 2µg/ml

Below the summary for the preparation of ATB:

Four 50 ml tubes were prepared for each antibiogram (ATB) (total 16 tubes):

- Tube 1: 6 ml of steril deionized water, 10-15 steril glass beads
- Tube 2: 9 ml of steril deionized water
- Tube 3: 19,8 ml of of steril deionized water
- Tube 4: 19,8 ml of of steril deionized water

Tube 1: a loopful of each *M. bovis* strain grown on LJ medium was suspended in 6 ml of distilled water. The clumps were reduced to obtain an homogeneous suspension which was vortexed for 30 seconds. The tube 1 was placed on horizontal shaker for 30 minutes and then under biosafety cabinet. The suspension was allowed to settle at room temperature for 5 minutes.

Tube 2: 1 ml of top layer was suspended from tube 1 to tube 2 and mixed carefully with the pipette.

Tube 3 : 0,2 ml of pellet was suspended from tube 2 to tube 3 and mixed carefully with the pipette. 1,2 ml of bacterial suspension (dilution 1/100) was removed and transfer in this way:

- 0,2 ml on LJ medium without antibiotic
- 0,2 ml on ethambutol-LJ medium
- 0,2 ml on rifampicin-LJ medium
- 0,2 ml on streptomycin-LJ medium
- 0,2 ml on isoniazid-LJ medium

The last 0,2 ml was transferred on tube 4.

Tube 4: the suspension was mixed carefully with the pipette. 0,2 ml of suspension was inoculated on LJ (dil 1/1000). The tubes were mixed carefully to uniform the distribution of inoculum on the entire surface of medium. The 6 tubes of LJ media were incubated with an inclination of 45 degrees at 37 °C until the complete absorption of inoculum (4-5 days). The tubes were observed 28 days after incubation and the colonies were counted on LJ dil. 1/100; if the

number was too high to be counted, the colonies were valued after a LJ dil. 1/1000.

Percentage resistance for each drug was calculated by dividing the total number of colony-forming units (CFU) on the drug- containing medium by the total number of colonies growing on the drug-free medium and multiplying the result by one hundred. A 1% standard cut-off value was used for the interpretation of resistance. Therefore, a culture with a percent resistance of less than 1% was considered susceptible to that particular drug at that concentration, while a culture with a percent resistance greater than or equal to 1% was considered resistant to that particular drug.

$$\frac{\text{Number of colonies on the drug quadrant}}{\text{Number of colonies on the control quadrant}} \times 100 = \% \text{ Resistance}$$

2.2.3.2 *Molecular detection of drug resistance: GenoType® Mycobacterium tuberculosis- multidrug resistantplus*

Several methods have been developed for the detection of mutations associated with drug resistance of MTBC. These include real time polymerase chain reaction (qPCR) (Lyn *et al.*, 2004) and commercially available PCR/line probe assays (Morgan *et al.*, 2005; Bang *et al.*, 2006).

These methods can be used to test positive cultures or smear- positive concentrated specimens for identification of MTBC and detection of mutations associated with resistance to INH and RMP. At present, molecular methods must be considered an adjunct to cultured-based methods and phenotypic culture-based drug susceptibility testing must be performed when a pure culture becomes available.

The GenoType® *M. tuberculosis- multidrug resistantplus* (MTBDR*plus*) VER 2.0 assay was used to perform drug susceptibility and detection of mutations in *rpoB*, *katG*, and *inhA* genes associated with resistance to RIF and INH on the 3 cases of light bacterial groths (table 5) according to the manufacturer's recommendations.

2.2.3.3 Susceptibility by Versatrek method

Moreover, the susceptibility testing to INH, STR, RFP, ETB of six bovine *M. bovis* isolates was performed by VersaTREK Myco susceptibility kit, VersaTREK Myco streptomycin kit and VersaTREK Myco PZA kit (Cleveland, OH, USA-formerly ESP Culture System, Difco, Detroit, USA), following the manufactures's protocol.

Appropriate inoculum sources were prepared starting from colonies grown on solid agar Stonebrink and/or Middlebrook 7H9 broth (tab. 4):

1. Cell suspension by Stonebrink medium: pure isolates (not more than 4-5 weeks old) growing on Stonebrink medium were scraped using a sterile loop and placed in a tube containing 5 ml sterile deionized water and glass beads. The tube was vortexed and allowed to settle for 15 minutes. Approximately 4/4,5 ml of top layer was removed, placed in a sterile test tube and adjusted to a No. 1 McFarland equivalent (approximately 3.0×10^7 CFU/ml) using sterile deionized water. The McFarland No. 1 from Myco was diluted 1:10 in deionized water.
2. Cell suspension by Middlebrook 7H9: five 4,5 ml tubes of Middlebrook liquid medium with 5 *M. bovis* beads were prepared and incubated at 37°C for 4 weeks. The dilution obtained was aseptically injected into the VersaTREK bottles as it was, without further dilution.

The purpose of using two different methods of preparation of the inoculum was to assess what could be the best and/or the faster to prepare. In principle, the susceptibility kit was inoculated with the solution coming from the colonies grown on Stonenbrink, while the Myco streptomycin kit and that of Pyrazinamide were prepared starting from the colonies grown on Middlebrook 7H9.

Where the concentration of one of the two solutions was too low, it was decided to mix the two preparations in order to obtain a single solution which was in conformity to the required concentration.

Table 4. Inocula (Stonebrink and/or Middlebrook 7H9) of the specimen prepared for susceptibility testing by Versatrek susceptibility kit, Versatrek myco streptomycin kit and Versatrek myco PZA kit and final drug concentration.

N°	Control of Susceptibility kit	RFP (µg/ml)	ISN (µg/ml)		ETB (µg/ml)		Control myco STR	STR (µg/ml)		Control Myco PZA	PZA (µg/ml)
			0,4	0,1	8	5		8	2		
		1	0,4	0,1	8	5		8	2		300
1	Stonebrink	Stonebrink	Stonebrink		Stonebrink		Stonebrink + Middlebrook 7H9	Stonebrink + Middlebrook 7H9(8 µg/ml)- Stonebrink (2 µg/ml)		Middlebrook 7H9	Middlebrook 7H9
2	Stonebrink	Stonebrink	Stonebrink		Stonebrink		Middlebrook 7H9	Middlebrook 7H9		Middlebrook 7H9	Middlebrook 7H9
3	Stonebrink	Stonebrink	Stonebrink		Stonebrink		Middlebrook 7H9	Middlebrook 7H9		Middlebrook 7H9	Middlebrook 7H9
4	Stonebrink + Middlebrook 7H9	Stonebrink + Middlebrook 7H9	Stonebrink + Middlebrook 7H9		Stonebrink + Middlebrook 7H9		Stonebrink + Middlebrook 7H9	Stonebrink + Middlebrook 7H9		Stonebrink + Middlebrook 7H9	Stonebrink + Middlebrook 7H9
5	Stonebrink	Stonebrink	Stonebrink		Stonebrink		Middlebrook 7H9	Middlebrook 7H9		Middlebrook 7H9	Middlebrook 7H9
6	Stonebrink	Stonebrink	Stonebrink		Stonebrink		Stonebrink	Stonebrink		Stonebrink + Middlebrook 7H9	Stonebrink + Middlebrook 7H9

The VTI/ESP system combines a liquid culture medium (VersaTREK Myco broth), a grow supplement (VersaTREK Myco GS) and a specific concentration of antibiotic (Table 4). Inoculated bottles with isolates of *M. bovis* are automatically incubated and continuously monitored (each 24 minutes) (Fig. 8). A VersaTREK Connector is attached to each bottle to establish a closed system for monitoring the bottle with the VersaTREK/ESP instrument. The sponges in the VersaTREK Myco bottles provide a growth support matrix and increase the surface area exposed to headspace oxygen. The technology of the VersaTREK/ESP Culture System II is based on the detection of headspace pressure changes within a sealed bottle due to microbial growth. A special algorithm has been developed for detection of very slow growing Mycobacteria.

With the VersaTREK/ESP Culture System II, anti-tuberculosis drugs can be injected into VersaTREK Myco bottles supplemented with VersaTREK Myco GS and specific *Mycobacterium tuberculosis* isolates in order to test their susceptibility or resistance to the chosen drugs. An inoculum of an isolated

Mycobacterium spp., prepared from colonies grown on solid media or liquid media, is added both to a drug-free control bottle, used as a positive control, and to a bottle containing the specific concentrations of drug. The presence of growth is determined automatically by the instrument, but the actual determination of susceptibility or resistance is performed by a manual comparison of the drug-free positive control bottle and the drug-containing bottles. If the organism is susceptible to a drug, either no microbial growth or delayed growth will be detected. If the organism is resistant to a drug, microbial growth will be detected within a specified time and is graphically observed on the instrument by the generation of a downward consumption curve.

Isolate information is entered into the system computer and the bottles are placed in the instrument for incubation at 35-37°C under stationary conditions. VersaTREK/ESP Culture System II detects mycobacterial growth by automatically monitoring (every 24 minutes) the rate of consumption of oxygen within the headspace of the culture bottle and reports that growth response with a positive signal. At the end of the specified incubation period, as determined by the drug-free control bottle for each isolate that is tested, the isolate is manually determined to be susceptible or resistant to a drug.

For susceptibility testing using the VTI/ESP system, a test isolate is interpreted as being susceptible or resistant to a drug based on a formula. The time to detection of the drug-free bottle is used to calculate susceptible or resistant interpretations using the following formula:

- Susceptible: An isolate in a drug- containing bottle that has not signaled within the three days detection period of the drug-free control is determined to be susceptible at that drug concentration.
- Resistant: An isolate in a drug- containing bottle is considered to be resistant at that drug concentration when its time to detection is equal to, or is within 3 days, rounded to the near whole number, of the time to detection of the drug-free control bottle.

All resistant results by Versatrek method on liquid medium should be

confirmed by an alternate method according to the manufacturer's instructions.

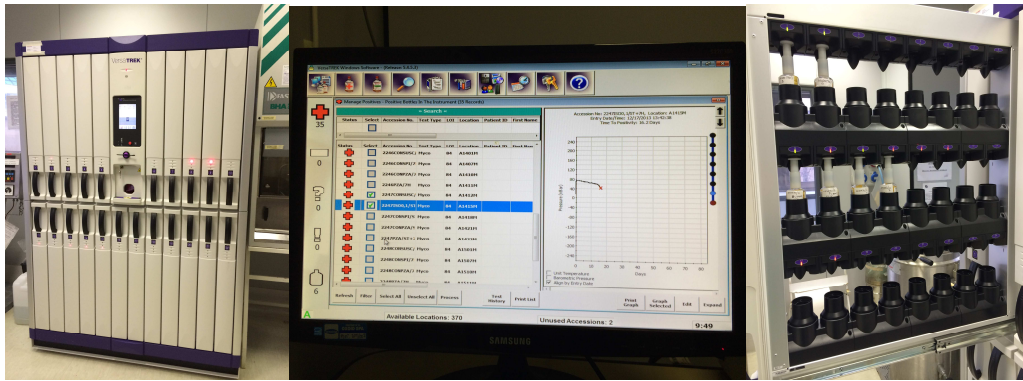


Fig. 8. The VersaTREK System machine

2.3 RESULTS AND DISCUSSION

There was no evidence of any drug resistance since all 24 *M. bovis* strains were susceptible to isoniazid, rifampicin, ethambutol, streptomycin by the proportion method. These results are supporting the lack of illegal antibacterial treatment applied to domestic animals which would provide selection pressure for the development of drug resistance. On the other hand, the absence of antibiotic resistant strains in wild boars suggests the absence of drug contamination in the environment, considering the feeding habits of wild boars, i.e. rooting, and the persistence of MTC bacteria in the environment for months (Duffield and Young, 1985; Fine *et al.*, 2011), which is a sufficient length of time to represent a risk of exposure for different species that share a habitat.

Light bacterial growth (table 5) was pointed out in isoniazid-medium (2 samples, one isolated from cattle and one from wild boar) and in rifampicin-medium (1 sample, isolated from wild boar) (Tittarelli *et al.*, 2012). Further analyses were performed on these isoniazid and rifampicin resistant clones, since the genetic mechanisms whereby *M. bovis* become resistant are not fully known, and in human medicine was observed that sub-populations naturally resistant may lead, after 4 o 5 months of monotherapy, to the complete sostitution of the susceptible population (Gillespie, 2002).

No mutations in *rpoB*, *katG*, and *inhA* genes associated with resistance to RIF and INH were detected in the 3 cases of light bacterial growth.

Table 5: Antimicrobial susceptibility results from Ligurian cattle and wild boars by proportion method on Lowenstein-Jensen (LJ) medium-

Host species	Agar proportion method	Light bacterial growth
Cattle	0/6*	1/6°
Wild boars	0/18	2/18
Total	0/24	3/24

* Number of resistant isolates over the total number of isolates tested

° Number of light bacterial growth strains over the total number of isolates tested

Tab. 6 Results of antimicrobial susceptibility tests on bovine *M.bovis* by Versatrek method on liquid medium

N°	Control of Susceptibility kit	RFP	ISN		ETB		Control myco STR	STR		Control myco PZA	PZA
		1 µg/ml	0,4 µg/ml	0,1 µg/ml	8 µg/ml	5 µg/ml		8 µg/ml	2 µg/ml		
1	P after 5,6gg	S	S	S	S	S	P after 3,5gg	S	S	P after 4,4gg	R (P after 4,1gg)
2	P after 4,5gg	S	S	S	S	S	P after 2,5gg	S (P after 7,1 gg)	R (P after 2,5gg)	P after 2,5gg	R (P after 2,5gg)
3	P after 7,2gg	S	S	S	S	S	P after 3,2gg	S	S	P after 3,5gg	R (P after 4gg)
4	P after 4,5gg	S	S	S	S	S	P after 3,5gg	S	S	P after 4gg	R (P after 4gg)
5	P after 4,1gg	S	S	S	S	S	P after 3,2gg	S	S	P after 4gg	R (P after 3,6gg)
6	P after 2,7gg	S	S	S	S	S	P after 2,7gg	S	S	P after 2,7gg	R (P after 2,7gg)

P= positive

S= sensitive

R=resistant

The results of antimicrobial susceptibility tests performed on bovine *M. bovis* strains by Versatrek method are reported in table 6. All the isolates were fully INH, RIF and EMB sensitive, except for natural pyrazinamide resistance. A single strain of bovine origin showed resistance to STR at lower concentration

(2 µg/ml) by Versatrek method, not confirmed by proportion method (table 7).

Table 7. Drug susceptibility test results from 2004 to 2010

Host species	Proportion method	Versatrek method	MTBDR^{plus} assay
Bovine	0/6*	1/6	0/1
Wild boar	0/18	nt ^o	0/2

* Number of resistant isolates over the total number of isolates tested.

^o nt = not tested

In spite of the fact that *M. bovis* infects humans, limited information about drug susceptibility is available. The susceptibility of isolates to four anti-tuberculous drugs commonly used for the therapy against tuberculosis in humans was tested. All the western Ligurian isolates were sensitive to INH, RIF, EMB and STR by the proportion method. These results would be in accordance with the ban of treatment of livestock as slaughter of animals that react positive to diagnostic test in the eradication campaigns is compulsory.

The lack of resistances in the western ligurian isolates from animal origin are in agreement with sensitivity to INH, RIF, EMB, STR, OFX in Spain (Romero *et al.*, 2007); to INH and RIF in the United Kingdom (Hughes *et al.*, 2003), to INH, RIF, STR, EMB in Brazil (Parreiras *et al.*, 2004) and INH, RIF, STR, EMB and quinolones in the United States (Daly *et al.*, 2006; Fitzgerald *et al.*, 2011).

However, they are in contrast with the high percentages of resistance to INH and RIF found in 2 other Italian studies (Sechi *et al.*, 2001; Cavirani *et al.*, 2003). This may be explained with differences in the study area (the former study was performed in Sardinia), or in the period of study and method applied, as the latter reported the susceptibility of *M. bovis* strains to antituberculous drugs (isoniazid and rifampin) detected by radiometric BACTEC 460TB system in the period 1995-1999. Since the results of this study indicate no antimicrobial resistance in the Western Liguria region, this is

a welcome finding for public health officials. However, the development of MDR strains of tuberculosis is a worldwide problem that has led *M. bovis* to be recognized as a reemerging pathogen (Gomez-Lus R *et al.*, 2000).

The World Health Organization (WHO) recommends the use of liquid culture techniques for TB diagnosis and drug susceptibility. The standard proportion method to assess the *M. bovis* susceptibility is the gold standard test (Canetti *et al.*, 1963), but alternative liquid culture systems (VersaTREK®MYCO susceptibility kit, streptomycin and PZA kit) should be applied for the rapid and timely detection of drug-resistance in tuberculosis strains. Technological advances in laboratory equipment such as automated broth-based culture systems have certainly reduced the time of detection of antibiotic resistant strains and may increase the total number of tests performed in a given time.

Our results seem to indicate that western ligurian *M. bovis* of animal origin are not the likely source of drug-resistant human cases, even though the study was carried out in a restricted area and on low number of strains. Therefore, the development of drug-resistant *M. bovis* would be caused in the human reservoirs because of inadequate therapy.

2.4 CONCLUSIONS

Although cattle represents the primary TB hazard (Hardstaff *et al.*, 2014), it's important the role that wildlife species have and, in particular there are likely to be significant advantages to TB control from developing integrated TB surveillance schemes incorporating both cattle and wild species.

In particular, wild boar represents a sensitive indicator of environmental contamination by *M. bovis* in Imperia province; considering wild boars feeding habits, i.e. rooting, the fact that no drug resistance was found in *M. bovis* strains isolated from these animals suggests the absence of drug contamination in the environment confirming their role of bioindicator.

On the other side, the absence of any drug resistance in cattle could be explained with the compulsory eradication program of bovine tuberculosis by elimination of infected animals and the ban on antituberculous drug treatments

in animals. However, there is the very real opportunity for other animals in the same herd -which may be harboring tuberculosis- to receive limited antibacterial therapy for other conditions, such as respiratory disease, mastitis, or other localized infections. This may lead to the insurgence of drug resistance in *M. bovis* strains despite all measures adopted for their control. In this type of environment, in fact, asymptomatic and undetected tuberculosis carriers will be subjected to similar selection pressures that have produced the current worldwide emergence of MDR and XDR tuberculosis strains in humans.

To strengthen the results of this study, we are planning to extend the survey to *M.bovis* strains isolated in the entire Liguria region, Piedmont and Valle d'Aosta region.

For what concerns the methods applied in this study, the proportion method to assess the *M. bovis* susceptibility to INH, RIF, EMB and STR is the gold standard, but the use of ancillary liquid culture systems (Versatrek method on liquid medium) should be developed for the rapid and timely detection of drug-resistance in tuberculosis strains. Microbiology and molecular techniques complement each other in the study of the drug-resistance and epidemiology.

In conclusion, due to the risk of *M. bovis* transmission from animal to humans and among human beings, the antibiotic resistance surveillance system is necessary to control the susceptibility of these species, and in this sense wild boar monitoring is very important in an area where the domestic/wildlife proportion is inverted.

The importance of this kind of studies can be found in the “One Health” concept, recognizing that the health of humans is connected to the health of animals and the environment.

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Annex 1

Research activity performed during the PhD study:

Articles published in international journals

Scaglione F.E., Bollo E., Pregel P., Chiappino L., Sereno A., Mignone W., Moschi R., Garibaldi F., **Tittarelli C.**, Guarda F. (2013). Heart pathologies in dolphins stranded along the northwestern Italian coast. *Diseases of aquatic organism*. 107 (1): 31-36.

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Contribution to conferences

11-12 October 2012 - IZS del Mezzogiorno - Portici, Italy

Talk at the “Corso di formazione Sorveglianza sanitaria dei cetacei spiaggiati lungo le coste delle regioni Campania e Calabria”. Talk title: Modalità di esecuzione dell’esame necroscopico e protocollo di campionamento in cetacei spiaggiati.

20 July 2012 – IZSPLV – Torino, Italy

Talk at the “La ricerca corrente dell’IZSPLV: a 360° per difendere la salute del territorio”. Talk title: Sorveglianza sanitaria dei cetacei spiaggiati lungo le coste della Liguria, della Toscana e del Lazio.

13 April 2012 - A.O.U. Maggiore della Carità di Novara, Italy

Talk at the “Corso: Tubercolosi umana ed animale: aggiornamento in materia di terapia, diagnosi e cura”. Talk title: Tubercolosi nel cinghiale: esperienze di campo e attività di ricerca dell’IZSPLV

3-4 November 2011 - Ministero della salute - Roma, Italy

Talk at the “Corso di formazione per referenti degli IZZSS nel quadro della

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Salogni C., Scaglione F.E., Bollo E., Chiappino L., Sereno A., Mignone W., Moschi R., Garibaldi F., **Tittarelli C.**, Guarda F. Cardiac pathology in Bluefin tuna (*Thunnus thynnus*). LXVII Convegno Nazionale S.I.S.Vet 17-19 Settembre 2013. Pag 6.

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Di Guardo G., Di Cesare A., Casalone C., Iulini B., Mignone W., **Tittarelli C.**, Otranto D., Proietto U., Castagna G., Buffatello G., Forster F., Kennedy S., Traversa D. (2011) Characterization of *Toxoplasma gondii* isolates from meningo-encephalitis affected striped dolphins (*Stenella coeruleoalba*). 25th Conference of the European Cetacean Society. 21st-23rd March 2011, Cadiz; Spain. Pag 285.

Casalone C., Iulini B., Pautasso A., **Tittarelli C.**, Pintore M.D., Corbellini D., Serracca L., Garibaldi F., Grattarola C., Gorla M., Fioravanti M.L., Varello K., Di Guardo G., Caramelli M., Mignone W. Laboratory findings in cetaceans stranded along the Ligurian sea coast of Italy (2007-2010). OIE Global Conference on Wildlife, Paris (France), 23-25 February 2011. Pag. 55.

Courses attended during the PhD study

11 December 2013 - IZSPLV- Torino, Italy

La ricerca degli *E. coli* produttori di verocitotossina (VTEC) nei germogli (Reg. 209/2013) e negli alimenti: prospettive e criticità legate all'interpretazione del dato analitico

04 April 2013 – IZSLT - Roma, Italy

Tubercolosi, brucellosi, leucosi bovina enzootica: valutazione dei costi dei piani di eradicazione nella regione Lazio

24-26 October 2012 – Sorrento, Italy

XIV congresso nazionale S.I.Di.L.V

10 February 2012 - Fondazione iniziative zooprofilattiche e zootecniche -

Brescia, Italy

Convegno Pipistrelli: biologia e malattie

11 January 2012 - Casinò di Sanremo, Italy

Convegno: la ricomparsa del lupo sulle Alpi liguri

26-27 September 2012 – Izsplv – Torino, Italy

Sanità pubblica veterinaria: impostazione di un piano di monitoraggio sanitario e di sorveglianza selvatica. Proposta realizzabile in Italia?

08 February 2012 – IZSPLV – Torino, Italy

Oliamm-lo strumento gestionale per gli approvvigionamenti Izsplva

28 October 2011 - Diano Marina, Italy

Giornata di aggiornamento sul randagismo

11-12 May 2011 - Asl Nuoro, Italy

Corso “Attività di sanità pubblica veterinaria nella gestione degli spiaggiamenti di animali marini

7 February 2011 – IZPLV – Torino, Italy

Corso base ADR (ediz.2)

22 November 2011-6 December 2011 – IZSPLV - Imperia, Italy

Evento formativo: la norma uni cei en iso/iec 17025:2005 e il manuale della qualità dell'izsplva. Dalla "grammatica" alla "pratica" (codice n. 8565 edizione 35)

28 November 2011 - IZSPLV, Torino, Italy

Evento formativo: Oliamm-lo strumento gestionale per gli approvvigionamenti IZSPLVA (codice n. 9103 edizione 2)

Participation in international meetings

28 August - 2 September 2011 - Zaragoza, Spain

Seventh Ticks and Tick-borne Pathogens International Conference.

4-5 July 2011 - University of Liege, Veterinary college, Belgium

5th International Cetacean Necropsy Workshop

21-23 March 2011 - Cadiz, Spain

25th Conference of the European Cetacean Society (ECS)

Participation in national research project

1. Sorveglianza sanitaria dei cetacei spiaggiati lungo le coste della Liguria, della Toscana e del Lazio- progetto di ricerca corrente finanziata dal Ministero della Salute Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti
2. Sviluppo di una rete di sorveglianza diagnostica a tutela della salute e de benessere dei cetacei spiaggiati lungo le coste del territorio nazionale- progetto di ricerca corrente finanziato dal Ministero della Salute Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti
3. Tubercolosi: aggiornamento in materia di diagnostica ed epidemiologia delle infezioni nelle specie animali, zootecniche, selvatiche e d'affezione- progetto di ricerca corrente finanziata dal Ministero della Salute Dipartimento per la Sanità Pubblica Veterinaria, la Nutrizione e la Sicurezza degli Alimenti

Partecipazione in internazionali progetti di ricerca

1. "RESEAU transfrontalier de lutte contre les moustiques nuisants et vecteurs de maladies"-progetto Italia-Francia marittimo 2007-2013

